Regulation of the Na^+-D-glucose cotransporter SGLT1 in small intestine in response to bariatric surgery and peptides derived from protein RS1 (*RSC1A1*)

Regulation des Na^+-D-Glucose Kotranporters SGLT1 im Dünndarm nach bariatrischen Operation und durch von Protein RS1 (*RSC1A1*) abgeleitete Peptide

Doctoral thesis
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1. Introduction

Obesity and type 2 diabetes mellitus (T2DM) are the major health and economic challenges in the modern societies that have raised concern worldwide. Globally it is estimated that 387 million people suffer from diabetes for a prevalence of 8.3% and the number may increase to 590 million by the year 2035 as per the International Diabetes Federation (Guariguata, Whiting et al. 2014).

The increasing prevalence, variable pathogenesis and complications of type 2 diabetes emphasise the urgent need for new treatment strategies. Alternative treatments targeting different models of this disease require careful and responsible examination. Bariatric surgery is one of the options for controlling diabetes in patients with high BMI-Body Mass Index. Different bariatric surgical procedures and a novel pharmacological approach for type 2 diabetes treatment were discussed in this study.

1.1 Bariatric surgery

Bariatric surgery represents the first-line treatment for morbid obesity, leading to improvement of obesity and Type 2 Diabetes mellitus. Despite extensive research to discover non-invasive methods for the treatment of diabetes, bariatric surgery remains the most effective long-term treatment. (Brolin 2002, Christou, Sampalis et al. 2004, Steinbrook 2004, Buchwald, Estok et al. 2009). It is a treatment option for patients with body-mass index (BMI, the weight in kilograms divided by the square of the height in meters) of at least 40 (NIH) and people with a BMI 35 with coexisting health problems such as type 2 diabetes, the risk of coronary artery disease and sleep apnea (Dixon, Zimmet et al. 2011). The surgery intends to create a physiological condition of malabsorption either by reducing or restricting gastric volume for glucose absorption. Malabsorptive and restrictive bariatric procedures include partial or total removal of the stomach; they alter regulatory mechanisms governed by the stomach such as regulation of appetite, secretion of bile acid and pancreatic enzymes. Several bariatric procedures performed in patients and tested in animals include partial removal of the stomach (sleeve gastrectomy)(Buchwald, Estok et al. 2009), bypass of the foregut (duodenal-jejunal bypass)(Jurowich, Rikkala et al. 2013), bypass of the foregut in combination with partial or total removal of the stomach (Roux-en-Y-gastric biliary bypass)(Cohen, Pinheiro et al. 2006), bypassing the duodenum and jejunum (biliopancreatic diversion)(Scopinaro, Gianetta et al. 1979) and transposition of a segment of the distal ileum

into the proximal jejunum (ileal transposition)(Koopmans, Ferri et al. 1984, Patriti, Aisa et al. 2007).

Bariatric surgery provides significant and sustained weight loss. Dramatic effect of this surgery on the improvement in type-2 diabetes has been seen as an additional outcome (Brolin 2002, Pories and Dohm 2009). Evidence provided that many obese, type 2 diabetic patients who underwent bariatric surgery have prolonged improvement in glycemic control (Heymsfield, Segal et al. 2000). Roux-en-Y Gastic Bypass (RYGB) is a commonly performed weight loss surgery that includes the resection of stomach to a small pouch along with duodenal and jejunal bypass. Morbidly obese patients with an associated type 2 diabetes who underwent RYGB show rapid improvement in glycemic control in approximately 80% of the cases (Schauer, Burguera et al. 2003). RYGB provides a rapid improvement in glucose regulation by enhanced insulin sensitivity and beta cell responsiveness to glucose. The glycemic control often occurs within days, long before significant weight loss (Rubino 2008). Unfortunately, many non obese type 2 diabetic people do not fulfil the NIH criteria (BMI > 35) to be eligible for bariatric surgery. RYGB also shows positive effect on type 2 diabetic people with a BMI < 35 (Cohen, Pinheiro et al. 2006). The results from previous studies suggest that type 2 diabetes might be an operable disease (Rubino, Forgione et al. 2006). Surgical techniques without resection of the stomach, such as duodenal-jejunal-bypass and ileal transposition have been developed to deconstruct the complex RYGB procedure.

1.1.1 Duodenal- Jejunal bypass (DJB)

Duodenal- Jejunal bypass (DJB) is an experimental metabolic procedure in which duodenum and (proximal) jejunum are bypassed without resection of stomach. Several groups have shown data indicating dramatic improvement in glucose homeostasis in obese and non obese animal model of type 2 diabetes after duodenal-jejunal exclusion (Rubino and Marescaux 2004, Rubino, Forgione et al. 2006, Kindel, Yoder et al. 2009, Speck, Cho et al. 2011). The mechanism of how the DJB induces immediate improvement in glycaemia independent of weight loss remains intriguing. According to “Foregut hypothesis” the effect of DJB on diabetes depends on exclusion of the duodenum and proximal jejunum from the transit of nutrients, possibly blocking the secretion of a putative signal that promotes insulin resistance and type 2 diabetes (Rubino and Marescaux 2004, Cummings, Overduin et al. 2005). Previous studies in rats suggested that the surgical rerouting of the small bowel after DJB lead to changes in gut hormones including glucagon-like peptide 1 (GLP-1) and glucose-

dependent insulinotropic peptide (GIP), which are involved in the regulation of insulin secretion (Rubino, Forgione et al. 2006, Patriti, Aisa et al. 2007, Bose, Olivan et al. 2009, Speck, Cho et al. 2011). Rubino et al in 2006 proposed that the modified release of gut hormones is an important mechanism for the improvement in glucose tolerance. However, results often conflict and no clear pattern has emerged, so the positive effects of surgery are generally not attained solely by the hormonal changes (Bose, Olivan et al. 2009). The exclusion of the proximal small intestine might decrease glucose uptake capacity, which might contribute to the improvement of glycemic control. However, the relative importance of these mechanisms remains poorly understood.

1.1.2 Ileal transposition (IT)

Ileal transposition (IT) is another bariatric surgical procedure performed by transposition of a segment of the distal ileum into the proximal jejunum avoiding any gastric resection or intestinal bypass. Previous studies in rats indicated that IT is one of the effective surgical procedures for non-obese type 2 diabetes (Koopmans, Ferri et al. 1984, Patriti, Facchiano et al. 2005). Ileal transposition is neither restrictive nor malabsorptive procedure but nevertheless produces dramatic improvements in glucose regulation independently of weight loss. Ileal transposition (IT), specifically designed to improve glycemic control, has so far shown great success in rodent studies (Koopmans, Sclafani et al. 1982, Koopmans, Ferri et al. 1984, Patriti, Aisa et al. 2007, Wang, Hu et al. 2008, Chelikani, Shah et al. 2010, Zhang, Wang et al. 2011, Gaitonde, Kohli et al. 2012, Grueneberger, Karcz-Socha et al. 2014).

Unlike other bariatric procedures, IT does not change the stomach volume, intestinal length and food passage. The “hindgut hypothesis” suggests that glycemic control results from the expedited delivery of nutrient chyme to the distal intestine, promoting a physiologic signal that improves glucose metabolism (Mason 1999, Cummings, Overduin et al. 2004). Enhanced stimulation of the lower gut is thought to play a key role in resolution of diabetes following Ileal transposition (IT). Further, Buchwald et al reported that the direct stimulation of the human terminal ileum and cecum by a food hydrolysate evokes significant plasma GLP-1 and PYY elevations (Buchwald, Dorman et al. 2014). Fast delivery of food to the terminal ileum is expected to be pathophysiologically responsible for improved glycemia after IT surgery. These results suggest that this surgical procedure may be suitable for the treatment of type 2 diabetes. Thus IT may be a good model for elucidating gut-related

mechanisms regulating glucose homeostasis, energy balance and metabolism to improve diabetic control by bariatric surgery.

It is still an important question which part of the gastrointestinal tract is the most important for glycemic control. While Ileal transposition (IT) allows the study of impact of direct lower gut stimulation, DJB on the other hand shows an insight on jejunal stimulation for glycemic control and body weight reduction. The possible mechanism behind the improvement of diabetes after IT may be due to the action of terminal ileum. However, the underlying mechanisms and the length of the ileum sufficient to elicit metabolic benefits after IT surgery are still elusive. It is unknown whether the control of diabetes is due to hormonal changes and/or associated morphological changes.

The anti-diabetic effect of DJB, ileal transposition (IT), and RYGB is mainly due to glucagon-like peptide 1 (GLP-1) and glucose-dependent insulin tropic peptide (GIP), which participate in the regulation of insulin secretion. The secretion of these gut hormones is triggered by D-glucose and other dietary nutrients (Koopmans, Ferri et al. 1984, Strader, Vahl et al. 2005, Wang, Hu et al. 2008, Chelikani, Shah et al. 2010, Nausheen, Shah et al. 2013). Hormone GLP-1 is secreted by L-cells, mainly located in the ileum. IT surgery significantly improves plasma glucose homeostasis with increased GLP-1 secretion (Buchwald, Dorman et al. 2014). The Na^+-D-glucose co-transporter (SGLT1), located in luminal membranes of enterocytes, K-cells, and L-cells, plays a key role in the absorption of D-glucose and glucose-dependent secretion of GIP and GLP-1 (Gorboulev, Schurmann et al. 2012). Since SGLT1 is an important factor in both these surgeries, it is necessary to understand the physiological impact of SGLT1 in its different locations in health and disease and after bariatric surgery procedures.

1.2 Sodium Dependent Glucose Co-transporter 1 (SGLT1)

SGLT1 is a member of the solute carrier family SLC5 encoded by the SLC5A1 gene (Hediger, Turk et al. 1989). It is a high affinity low capacity transporter (Hediger and Rhoads 1994) localized at the apical plasma membrane in the epithelial cells of the small intestine and the kidney. SGLT1 is expressed mainly in the intestine, to a lesser extent observed in the kidney (in the late part of renal proximal tubule), liver, lung, heart, and the parotid and submandibular salivary glands (Balen, Ljubojevic et al. 2008, Wright, Loo et al. 2011, Vrhovac, Balen Eror et al. 2014).

SGLT1 plays a critical role in intestinal glucose transport. Monosaccharides such as glucose produced during the digestion of complex carbohydrates forms one of major nutrients in diet and are a major source of energy for mammals. Entry of glucose into the intestinal epithelial cells (enterocytes) is mediated by active SGLT1 transporter on the apical surface (Hediger, Coady et al. 1987). Glucose is then transported into the blood stream by the facilitative glucose GLUT2 transporter located in the basolateral surface (Wood and Trayhurn 2003). The translocation of D-glucose is mediated by a secondary active co-transport system, driven by a sodium gradient generated by the Na^+/K^+ ATPase system (Wright, Loo et al. 1994, Wright, Hirayama et al. 2007). The Na^+/glucose cotransporter (SGLT1) couples sugar transport to Na^+ gradients across the intestinal brush border with a stoichiometric ratio of 2:1 (Wright, Loo et al. 1994). Defects observed in SGLT1 trafficking and function lead to glucose-galactose malabsorption syndrome (Wright 1998).

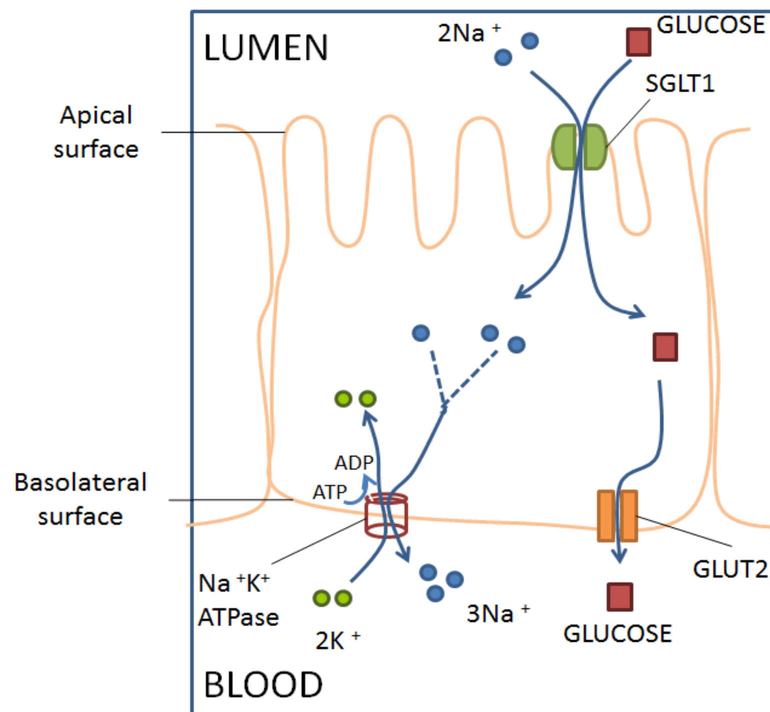

Figure 1: A model for glucose absorption across the small intestine: SGLT1 is the sodium dependent glucose transporter on the brush border membrane (BBM). Glucose is co-transported along with Na^+ across the brush border membrane by SGLT1, and the Na^+ is then transported out across the basolateral membrane by the Na^+/K^+ pump. Glucose accumulates within the cell and then diffuses out into blood across the basolateral membrane through GLUT2. Modified from Wright et al., 2004.

1.2.1 Regulation of SGLT1 by RS1 protein

RS1 protein is encoded by the intronless single copy gene *RSC1A1*. It is a 67-68 Kilo Dalton protein, specific to mammals, found in human (Lambotte, Veyhl et al. 1996), pig (Veyhl, Spangenberg et al. 1993), rabbit (Reinhardt, Veyhl et al. 1999), and mouse (Osswald, Baumgarten et al. 2005). RS1 is an intracellular protein which shows broad tissue distribution. It is expressed in intestine, kidney, liver, neuron and some extent in lung and spleen (Veyhl, Spangenberg et al. 1993, Lambotte, Veyhl et al. 1996, Poppe, Karbach et al. 1997, Reinhardt, Veyhl et al. 1999). Studies showed that RS1 is associated with the plasma membrane, around the Trans Golgi network and within the cell nucleus (Veyhl, Spangenberg et al. 1993, Lambotte, Veyhl et al. 1996, Valentin, Kuhlkamp et al. 2000, Osswald, Baumgarten et al. 2005, Kroiss, Leyerer et al. 2006).

SGLT1 dependent glucose absorption is regulated by various factors to meet the physiological demand of the body (Ferraris 2001). RS1 is involved in the regulation of some

plasma membrane transporters that belong to different protein families such as SGLT1, organic cation transporters (OCT), and concentrative nucleoside transporters (CNT) (Lambotte, Veyhl et al. 1996, Errasti-Murugarren, Fernandez-Calotti et al. 2012). Among these, SGLT1 has been studied extensively because of its physiological importance. Increase of SGLT1 expression and D-glucose absorption in small intestine was seen after removal of RS1 (Osswald, Baumgarten et al. 2005). Down-regulation of SGLT1 activity was seen upon injecting hRS1 protein in hSGLT1 expressing *Xenopus laevis* oocytes (Veyhl, Keller et al. 2006). Thus the protein RS1 was identified as a physiological target for SGLT1 regulation.

Several functional domains of the RS1 protein have been identified. Among these an N-terminal domain (16-98 and 15-92 amino acids in human and mouse respectively) is responsible for the posttranscriptional down-regulation. This short term down-regulation of SGLT1 was due to the inhibition of release of SGLT1 vesicles at the Trans Golgi network (TGN) (Veyhl, Wagner et al. 2003, Kroiss, Leyerer et al. 2006, Veyhl, Keller et al. 2006). Peptide sequences Gln-Ser-Pro (QSP) (Vernaleken, Veyhl et al. 2007) and an octapeptide Ser-Asp-Ser-Asp-Arg-Ile-Glu-Pro (SDSDRIEP) (M. Vehyl-Wichmann, et al data unpublished) were identified capable of regulating SGLT1 activity in oocyte system. A tripeptide Gln-Cys-Pro (QCP) was also identified in the C-terminal sequence of hRS1 that regulates of hSGLT1 post-transcriptionally (Vernaleken, Veyhl et al. 2007). Both tripeptides QSP and QCP were identified as high affinity inhibitors of hSGLT1 post transcriptionally (Veyhl, Keller et al. 2006).

QCP and QSP work at low glucose concentrations, but not at high concentrations of sugar (Vernaleken, Veyhl et al. 2007). By modifying QSP, a tripeptide (QEP) was developed. QEP (Gln-Glu-Pro) is a phosphorylation mimicking variant of QSP. In contrast to QCP and QSP, QEP down-regulated SGLT1 activity even at high sugar concentrations. In oocyte experiments performed by Maike Veyhl-Wichmann, the affinity of QEP to down-regulate human SGLT1 expression was higher, in comparison with QSP (Unpublished data). Since SGLT1 regulation plays an important role in glycemic control, these peptides can be used for the treatment of metabolic mismanagement disorders e.g. Diabetes mellitus. However, the importance of RS1 tripeptides and the effects of RS1 phosphorylation for SGLT1 regulation have only been depicted from *in vitro* studies (Experiments carried out in *Xenopus laevis* oocytes). Further investigation is necessary to understand the function of RS1 tripeptides and its phosphorylation in regulation of SGLT1 by *in vivo* studies. QEP, QSP and thiophosphorylated QSP (QSpP) were the RS1 derived tripeptides considered in this study to

understand the regulation of SGLT1. This might impart knowledge for the better management of carbohydrate metabolism.

2. Aim of the study

The aim of this study was to elucidate the role of different bariatric surgical procedures (DJB and IT) and RS1 derived peptides on the regulation of small intestinal SGLT1 mediated D-glucose transport. For this, it is necessary to consider initially the complex regulation of SGLT1 in small intestine. To address this, the influence of other factors on SGLT1 regulation such as removal of RS1, gender and diabetic disease state was investigated. DJB and IT in rats with experimental type 2-like diabetes resulted in an increase in the secretion of enterohormone GLP-1 which is involved in insulin regulation. Since SGLT1 is involved in the secretion of GLP-1, we wanted to determine whether and how SGLT1 transport activity is regulated upon these surgical procedures. We also wanted to investigate the morphological changes in the mucosa and the expression of SGLT1 protein in the enterocytes after IT surgery by immunohistochemical analysis.

To further elucidate SGLT1 regulation, we studied the effect of RS1 derived tripeptides on SGLT1 activity. To determine whether oral application of these peptides can be used for downregulation of SGLT1 mediated glucose absorption after glucose rich meals, we investigated the regulation of different RS1 derived tripeptides in mouse and human jejunal tissues (*ex vivo)* at high glucose concentrations. Later, we aimed at identifying the tripeptides that act as high affinity inhibitors of SGLT1-mediated glucose absorption. To establish the pharmacological significance of QEP which showed high affinity for SGLT1 inhibition, the tripeptide was studied at different concentrations as well as in different animal models such as pig, rabbit and mice (wild type, RS1 KO and Leptin KO mice).

3. Materials & Methods

3.1 Materials

3.1.1 Chemicals

All laboratory chemicals used were of pro analysi grade and purchased from Carl Roth GmbH (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), Perkin Elmer (Darmstadt, Germany) or AppliChem (Darmstadt, Germany).

3.1.2 Animals

Male Lewis rats weighing 180-200g, 7 weeks old, from Harlan (Venray, The Netherlands) were maintained in groups of 2-4 animals per cage in laminar flow hoods in a pathogen-free environment. The rats had free access to food and water. The study was reviewed and approved by the Animal Care Committee of the local government in accordance with the national guidelines for animal care (German Law for the Protection of Animals).

Mice were used according to Institutional guidelines and German laws. Wild type mice and RS1 KO mice of 129/OLA/C57BL/6 or C57BL/6 background was used for studies. Male mice of age 2-3 months were chosen for study. All animals were housed in a temperature controlled environment with a 12h-light/12h-dark cycle with free access to specific diet and tap water.

Rabbits were also used in the study. They were obtained from Institute of Anatomy and Cell Biology, University of Wurzburg and maintained in cages (one/cage) and given free access to food and water.

Pig intestinal tissues were collected from slaughter house, Rimpar, Wuerzburg.

Human jejunal tissues were provided by Dr. C. Jurowich, Center for Operative Medicine, University Hospital, Wurzburg. Human Jejunal tissue which is routinely removed during bariatric surgical procedure was collected in to ice-cold Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% (v/v) foetal calf serum (FCS, Sigma-Aldrich), 1% L-glutamine (PAA, Pasching, Austria), and 1% penicillin/streptomycin (PAA) and 5 mM D-glucose and transferred to the laboratory. All the patients were obese (BMI > 35) and aged between 40 and 60. The ethics application was made by Dr. C. Jurowich accordance with the ethical guidelines of the University of Würzburg.

3.1.3 Diets

Rats received a standard rodent diet (STD; Altromin No. 1324), a hypercaloric diet HFD (Altromin No. 40003), and a hypercaloric HFD mono- and disaccharides free diet (HFD-MF, Altromin No. 4006) which were obtained from Altromin GmbH, Lage, Germany. STD contained 19.5% protein, 4.1% fat, 29.2% polysaccharides, and 5.2% disaccharides. HFD contained 18.2% protein, 22.1% fat, 33.5% polysaccharides, 9.8% disaccharides and 1.7% monosaccharides. HFD-MF contained 18.2% protein, 22.1% fat, and 42.5% polysaccharides. The values are given as percentage of wet weight.

Mice were fed with standard normal diet (ND; Ssniff V1534-000R/M-H, 10mm). The ND was purchased from Spezialdiäten GmbH, Soest, Germany. Normal diet contained 36.4% starch, 19% protein, 4.9% fibre, 4.7% mono and disaccharides, 3.3% fat, minerals and vitamins.

3.1.4 Buffers and solutions

All the buffers were prepared by using deionised water. Compositions of the different buffers and solutions are given in corresponding section.

3.1.5 Radioactive substrate

[^{14}C] radiolabelled Methyl-α-D-Glucopyranoside (AMG), [glucose-^{14}C (U)]; (11.1 Gbq/mMol) was purchased from American Radiolabelled Chemicals, Inc. The total working concentration of AMG used for uptake method was 10 µM.

3.1.6 Antibodies used in this work

Primary antibodies: Polyclonal antibodies against amino acids 585-600 (PKDTIEIDAEAPQKEK-C) of rat SGLT1 (rSGLT1-Ab) were raised in rabbits. Antibodies against amino acids 581-599 (QEGPKETIEIETQVPEKKK-C) of the hSGLT1 protein (hSGLT1-Ab) were also raised in rabbits. The antibodies were affinity-purified using the respective antigenic peptide. Polyclonal antibody against glucagon-like peptide 1 (C-17; sc-7782; GLP-1-Ab) raised in goat was obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

Secondary antibodies: Cy3-labeled goat anti-rabbit IgG (GARCY3) obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and Alexa Fluor 488-labeled chicken

anti-goat IgG (CAG-488F, A21467) obtained from Life Technologies (Darmstadt, Germany), were used in this study.

3.1.7 Synthetic tri-peptides

Synthetic tripeptides QSP (Gln-Ser-Pro), thiophosphorylated QSP (Gln-Ser-Pro), QEP (Gln-Glu-Pro), QDP (Gln-Asp-Pro), QTP (Gln-Thr-Pro), QMP (Gln-Met-Pro), QIP (Gln-Ile-Pro), QVP (Gln-Val-Pro) and QAP (Gln-Ala-Pro) were obtained from the lab of Dr. Rüdiger Pipkorn, German Cancer Research Center, Heidelberg, Germany. Different concentration of peptides was used in the study.

3.1.8 Software

PRISM (GraphPad software, Inc., San Diego, Calif.) software was used for statistical analysis. Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA was used during quantification of Immunohistochemistry data.

3.2 Methods

3.2.1 AMG uptake in everted small intestinal rings

Rats/Mice, starved for 18 hours, were sacrificed between 11 a.m. and 12 noon. The small intestines were removed immediately and perfused with Krebs-Ringer buffer (25 mM HEPES, 108 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, pH 7.4, 37°C) to wash the intestine clean. The Intestine was everted using a steel rod and segregated in to Duodenum, Jejunum and Ileum. The parts were cut in to 1 cm length segments. The segments were incubated for 2 minutes at 37°C with Krebs-Ringer buffer containing 10 µM of SGLT1 specific glucose analogue [^{14}C]α-Methyl-D-glucopyranoside (AMG), with or without 0.2 mM of SGLT1 specific inhibitor phlorizin. Uptake was stopped by transferring the segments in to ice cold Krebs-Ringer buffer containing 0.2 mM phlorizin for 5 minutes. The segments were washed and kept in vials containing tissue Solubilizer Soluene-350 (Perkin Elmer Inc, Waltham, MA) for 12 hours at room temperature and 1 hour at 60°C. 200 µl of dissolved sample was taken in duplicates and mixed with 1ml of scintillation fluid-Lumasafe scintillation cocktail (Lumac LSC, Groningen, The Netherlands). The radioactivity of the dissolved samples was measured using Beckman Coulter TM LS 6500 multi-purpose scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

3.2.2 AMG uptake in human jejunal mucosa layer circle areas

Human jejunal tissues were obtained during bariatric operations. Tissues were carried in the ice cold DMEM and were washed with Krebs-Ringer buffer (25 mM HEPES, 108 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM CaCl2, pH 7.4, 37°C). Mucosal layer was removed and pieces of the mucosa with an identical surface area were isolated using a punching instrument. The pieces of mucosa were incubated for 30 min without tripeptides (control) and with indicated RS1 derived peptides in presence of 5 mM glucose. The pieces of mucosa were washed three times with Krebs-Ringer buffer at room temperature. The segments were incubated for 2 minutes at 37°C with Krebs-Ringer buffer containing 10 µM of SGLT1 specific glucose analogue [^{14}C]α-Methyl-D-glucopyranoside (AMG), with or without 0.2 mM of SGLT1 specific inhibitor phlorizin. Uptake was stopped by transferring the segments in to ice cold Krebs-Ringer buffer containing 0.2 Mm phlorizin for 5 minutes. The segments were washed and kept in vials containing Tissue Solubilizer, Soluene-350 (Perkin Elmer Inc, Waltham, MA) for 12 hours at room temperature and 1 hour at 60°C. 200 µl of dissolved sample was taken in duplicates and mixed with 1ml of scintillation fluid-Lumasafe scintillation cocktail (Lumac LSC, Groningen, The Netherlands). The radioactivity of the dissolved samples was measured was measured using Beckman Coulter TM LS 6500 multi-purpose scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

3.2.3 AMG uptake in rabbit and pig jejunal mucosa layer circle areas

Rabbits, starved for 18 hours, were sacrificed between 11 a.m. and 12 noon. Small intestines were taken immediately and perfused with Krebs-Ringer buffer. The intestines were opened and placed on Teflon plate. Pieces of the mucosa with an identical surface area were isolated using a punching instrument. Later, the same procedure was followed as mentioned above in case of human intestine tissue segments.

Pig jejunal tissue was obtained from slaughter house. It was carried in ice cold DMEM medium containing 5 mM glucose. After washing with Krebs-Ringer buffer, intestine was opened and placed on Teflon plate. Pieces of the mucosa with an identical surface area were isolated using a punching instrument. Again, the same procedure was followed as mentioned above in case of human intestine tissue segments.

3.2.4 Immunohistochemical analysis

Intestinal tissues collected from rat and human were washed with PBS-Phosphate buffered saline (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) at 4°C for 5 minutes. The segments were placed in fixation solution (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4, 4% (w / v) paraformaldehyde) overnight at 4°C. The following day segments were washed 3 times in PBS. Tissue storage was done in PBS with 0.02% sodium azide (NaN_3).

Tissues were incubated in 30% sucrose solution (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4, 30% (w/v) sucrose) overnight at 4°C. Later they were kept on tissue base mold, embedded in Tissue-TeK (Sakura,Japan), and frozen at -25°C. 4 µm thick cryosections were cut in a Leica CM 1850 cryostat (Leica Instruments, Nussloch, Germany). Sections were placed on Superfrost/Plus microscope glass slides, dried at room temperature for 4-5 hours, and kept at 4°C until further use.

Antigen exposure steps were performed prior to the administration of the primary antibody. The tissue sections were rehydrated in PBS for 15 minutes, followed by boiling in 10 mM citrate buffer, pH 6 in a microwave at 800 W for four cycles five minutes each. Between each cycle citrate buffer was refilled. Sections were kept in the same citrate buffer for 20 minutes so that it was allowed to cool to room temperature. This was followed by three washes five minutes each in PBS, incubation in 0.5% (v/v) and 2% (v/v) Triton X-100 in PBS for 15 and 30 minutes respectively. The sections were then washed three times for five minutes each in PBS and then blocked with 1% (w/v) fraction BSA- Bovine Serum Albumin solution in PBS for 30 minutes. Then the sections were incubated with primary antibody (1:500) overnight at 4°C. Later the sections were incubated in 0.1% (v/v) Triton X-100 in PBS for ten minutes and washed twice with PBS for five minutes. The secondary antibody (GARCY3) was diluted 1:800 with storage buffer, added to the sections and incubated for one hour at room temperature. It was followed by a further incubation with 0.1% (v/ v) Triton X -100 in PBS for ten minutes and two five-minute washes with PBS. For immunostaining with GLP-1-Ab, sections were similarly processed with GLP-1-Ab and the secondary antibody CAG-488F. After staining, the sections were covered with VECTA Shield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA), cover slipped and sealed with nail polish. After drying, the sections were examined with Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany) and the photos were taken using a

software-guided camera Spot RT Slider (Diagnostic Instruments, Sterling Heights,MI, USA). Rat intestinal tissues stained with (rSGLT1-Ab) were photographed at 20x magnification with a Keyence Biorevo BZ-9000 Microscope (Keyence Corporation, Osaka, Japan). The full focus function of the BZ-II Image Analysis application was used to merge captured images into a single image.

3.2.5 Quantification of immunostaining

Quantification was performed with Image J software supplied by the National Institute of Health (Bethesda, Maryland, USA). To compare staining of the BBM in different samples, fluorescence images with higher magnification were used. Staining of the BBM was quantified from the images. The obtained staining intensities were normalized to the length of the analyzed bands which was determined in parallel. The total length of BBM per cross-section, heights of the individual villi and width of the villi were determined using straight and/or free hand line function of Image J software.

3.2.6 Statistical analysis

AMG uptake measurements in everted segments of small intestine were performed at least in three animals for every condition. Mean $\pm$ SEM values were considered during statistical analysis. Significance of difference between SEM values of two groups was calculated using one sided student’s unpaired t-test. Significance of difference between SEM values of three or more groups were tested using one way Analysis of variance (ANOVA) with post hoc Tukey comparison, Significance levels are indicated as: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

4. Results

In this study, I intended to investigate the impact of different bariatric surgical procedures such as duodenal-jejunal bypass and ileal transposition on rat intestinal SGLT1 transport activity. I also investigated the transport activity of SGLT1 in the small intestine of mouse (Wild type and RS1 KO), rat and human with respect to conditions of gender and diabetic disease states.

In addition, the effect of RS1 on SGLT1 regulation was studied *ex vivo* using RS1 derived tri-peptides.

4.1 Transport activity of SGLT1 under different conditions of study

4.1.1 SGLT1 activity pattern in different regions of mouse intestine

SGLT1 plays key role in intestinal absorption of glucose. Although SGLT1 has been studied well, the detailed distribution pattern throughout the intestine has not been fully elucidated.

To investigate the SGLT1 activity pattern in different regions of intestine, SGLT1 dependent, phlorizin inhibitable, uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in different regions of mouse intestine (i.e. duodenum, jejunum, ileum, colon and rectum).

The result (Figure 2) suggests that SGLT1 follows a gradient pattern for glucose absorption at different regions of the small intestine in mice. Duodenum has higher SGLT-1 activity, which gradually decreases with jejunum, ileum, colon and rectum. This study represents an SGLT1 dependent activity for glucose absorption to be present also in the large intestinal region.

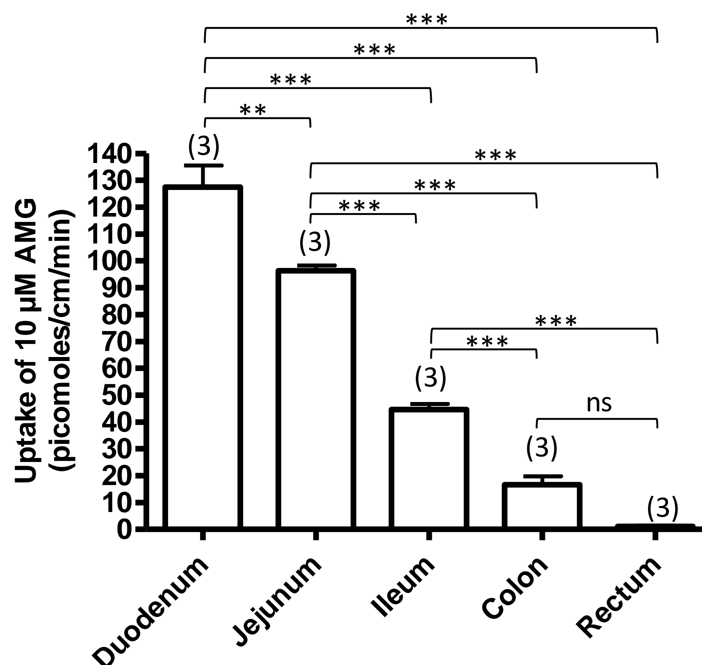

Figure 2: SGLT1 activity in different regions of mouse intestine: Wild type mice were starved for 18 hours before measuring SGLT1 mediated AMG uptake. Phlorizin inhibitable uptake of 10μM AMG, labelled with tracer amount of [^{14}C]AMG was measured in different regions of intestine. Uptake was calculated in terms of picomoles/cm/minute. The numbers of employed animals are shown in parenthesis. SGLT1 transport activity in different regions was compared. SGLT1 activity shows higher to lower gradient from duodenum to rectum. Significances of differences were calculated by ANOVA with post-hoc Tukey comparison (ns-not significant, **P < 0.01, ***P < 0.001).

4.1.2 SGLT1 activity in different regions of wild type and RS1 KO mice intestine

Previously it has been described that mice in which RS1 was removed (RS1 KO) compared to wild type mice show higher amount of SGLT1 immunoreactivity in the brush border membrane (BBM) of jejunum and in plasma membrane enriched (PME) fractions of jejunum (Osswald, Baumgarten et al. 2005). Because we observed different expression and activity of SGLT1 in different small intestinal regions of wild type mice (Figure 2), I investigated whether the difference between wild type and RS1 knockout (RS1 KO) mice is also observed in duodenum, ileum, colon and rectum.

SGLT1 activity in different regions of wild type and RS1 KO mice intestine was compared by measuring AMG uptake in everted small intestinal rings. The data (Figure 3) indicate that AMG uptake in RS1 KO mice jejunum is significantly higher (P < 0.001 for difference) than

in wild type mice jejunum, whereas the uptake in duodenum, ileum, colon and rectum were not significantly different between wild type and RS1 KO mice.

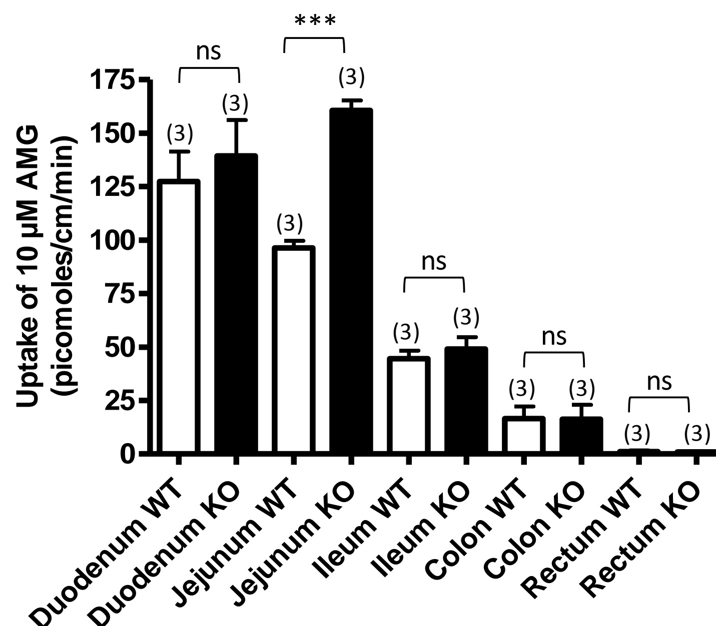

Figure 3: Comparison of SGLT1 activity in different regions of wild type and RS1 KO mice intestine: Wild type (open columns) and RS1 KO (closed columns) mice were starved for 18 hours before performing SGLT1 mediated AMG uptake. Phlorizin inhibitable uptake of 10 µM AMG was measured in different regions of intestine. Uptake was calculated in terms of pico moles/cm/minute. The numbers of employed animals are indicated in parenthesis. Regional difference was observed in both wild type and RS1 KO mice and RS1 KO mice jejunum showed significantly higher SGLT1 transport activity compared to wild type jejunum (ns-not significant, ***$P < 0.001$ for difference between Jejunum WT and Jejunum KO, ANOVA with post-hoc Tukey).

4.1.3 Gender based analysis of SGLT1 activity and protein expression at the BBM of small intestine

To analyze gender based SGLT1 activity, glucose absorption pattern was studied in everted jejunum of male and female mice. This study was also performed in obese male and female patients. Human jejunal tissues were obtained during bariatric operations. Identical areas of the dissected mucosa were used for the measurements. Phlorizin inhibitable uptake of 10 µM AMG, labelled with tracer amount of [^{14}C]AMG was measured in both mouse and human jejunal tissues. To study the difference in SGLT1 protein amount at the BBM, immunostaining of the BBM was compared between obese human male and female patients.

The result suggests that SGLT1 activity is not dependent on gender as no significant differences in phlorizin inhibited uptake of AMG were observed (Figure 4A and B). Immunohistochemical analysis revealed that the protein amount at the BBM is also not changed between male and female (Figure 4C). The data indicate that SGLT1 activity and protein expression is independent of gender.

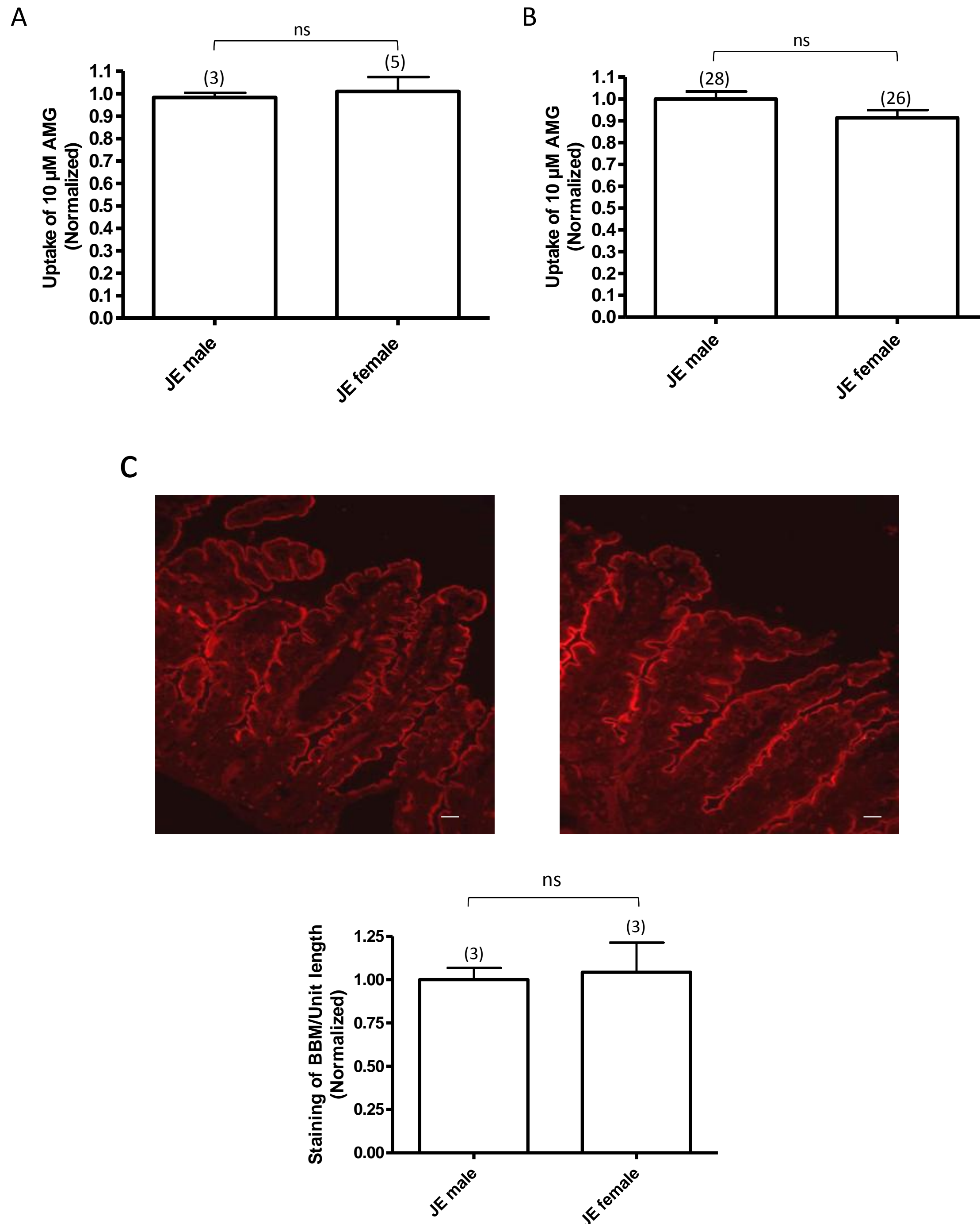

Figure 4: Comparison of SGLT1 activity and immunostaining of the BBM between male and female: SGLT1-mediated AMG uptake comparison between male and female mice (A) and humans

(B) is shown in this figure. Segments of everted jejunum from mice or identical areas of human jejunal mucosa were taken. Phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. No difference in SGLT1 activity was observed between male and female (P = 0.077). Immunohistochemical comparison of SGLT1 protein at the BBM of male and female human jejunum (C). Human jejunal tissue samples were collected from obese human male and female patients. Immunostaining was performed with hSGLT1-Ab. The fluorescence intensity of the BBM per unit length was calculated and normalized to 1 (15 determinations each). The numbers of employed human jejunal tissue samples are indicated in parenthesis. No difference in SGLT1 fluorescence intensity was found between male and female (ns- not significant, unpaired t-test). Bar: 20 µm.

4.1.4 Effect of type 2 diabetes on SGLT1 activity and protein expression at the BBM of small intestine

It has been reported that rats with experimentally induced type-1 diabetes mellitus exhibit increased SGLT1 mRNA and protein in enterocytes. (Debnam, Karasov et al. 1988, Burant, Flink et al. 1994). To determine the effect of type 2 diabetes on transport activity of SGLT1, I compared phlorizin inhibited AMG uptake mediated by SGLT1 in the jejunum of normal rats and of rats with T2DM. T2DM was induced by high fat diet and one application of low dose (30 mg/kg body weight) of streptozotocin. AMG uptake was measured in segments of everted small intestinal rings. Previous studies reported that in patients with T2DM, SGLT1 mediated glucose transport and SGLT1 protein in brush border membrane (BBM) vesicles isolated from duodenal biopsies was increased (Dyer, Wood et al. 2002). In our study I investigated the effect of type 2 diabetes in human intestinal SGLT1 activity and protein expression at the BBM. I compared SGLT1 protein expression and activity by immunohistochemical analysis and uptake measurements in jejunal mucosa from obese non-diabetic patients and obese type 2 diabetic patients.

Figure 5A shows that STZ induced induction of diabetes in rats on HFD resulted in an increased SGLT1 dependent glucose uptake. Figure 5B and C indicate that jejunal mucosa of obese patients with type 2 diabetes showed higher SGLT1 activity and higher amounts of SGLT1 protein at the BBM.

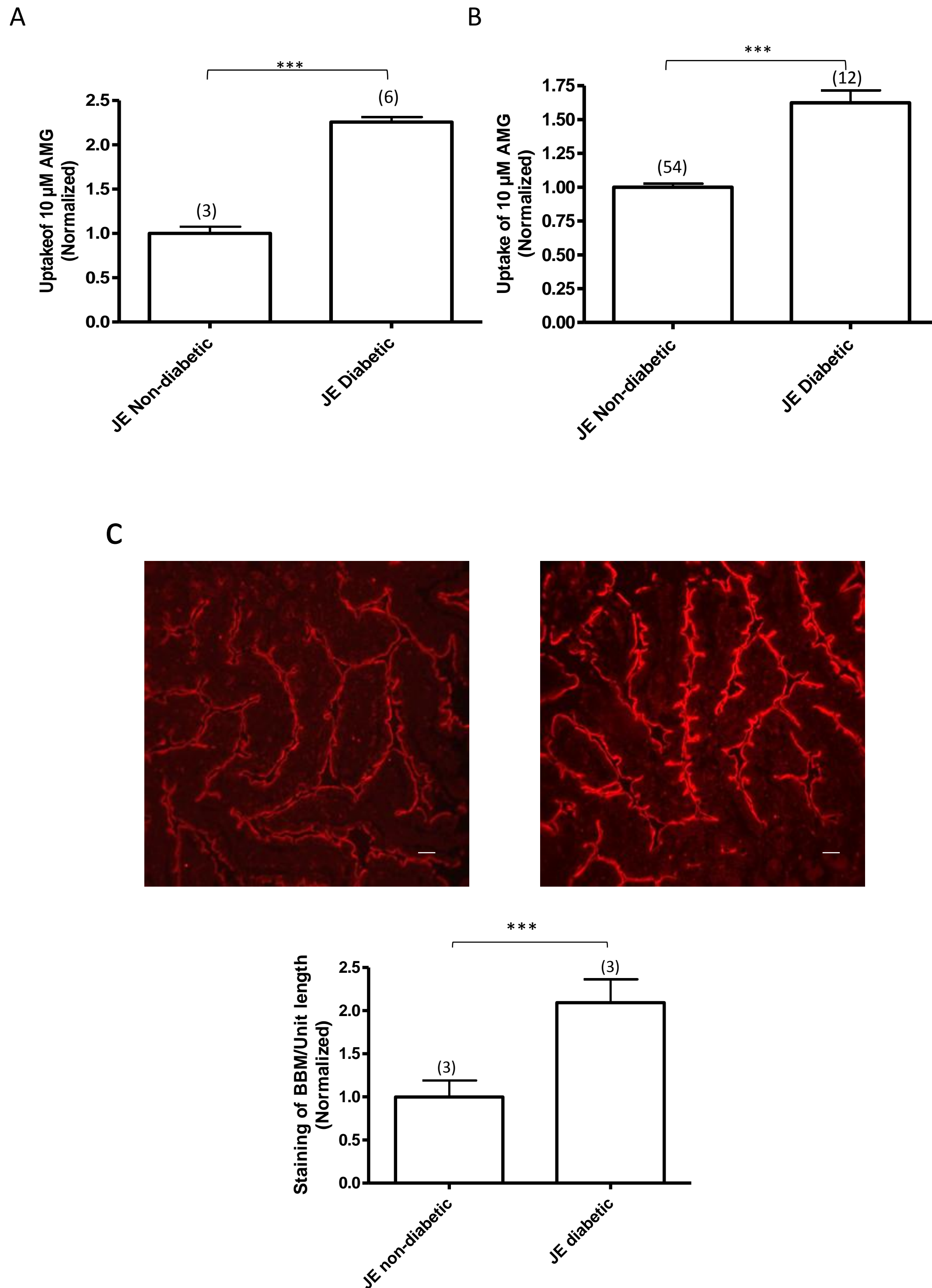

Figure 5: Comparison of SGLT1 activity and immunostaining of the BBM between non-diabetic and type 2 diabetic subjects: SGLT1-mediated AMG uptake comparison between non-diabetic and diabetic rats (A) and human (B) is depicted in this figure. Segments of everted jejunum from rats or identical areas of human jejunal mucosa were taken. Phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. The numbers of employed animals or human jejunal

tissue samples are mentioned in parenthesis. Significant increase in SGLT1 activity was observed in diabetic jejunal segments (***P < 0.0001). Immunohistochemical comparison of SGLT1 protein in the BBM of non-diabetic and diabetic human jejunum (C). Human Jejunal tissue samples were collected from obese non-diabetic and diabetic patients during bariatric operations. Immunostaining was performed with hSGLT1-Ab. The fluorescence intensity of the BBM per unit length was calculated (12 determinations each) and normalized to 1. Significant increase in SGLT1 fluorescence intensity was observed in diabetic jejunal segments (***P < 0.001 for difference, unpaired t-test). Bar: 20 µm.

4.2 Impact of different bariatric surgical procedures on rat small intestinal SGLT1

Duodenal-jejunal bypass (DJB) and ileal transposition (IT) were shown to improve oral glucose tolerance in rats on HFD with STZ induced type 2 like diabetes (T2LD). Because SGLT1 (localized in the BBM of small intestinal enterocytes) is rate limiting for intestinal glucose absorption (Gorboulev, Schurmann et al. 2012), it is important to know whether the function of SGLT1 is altered by these surgical procedures and whether an altered glucose absorption contributes to the improvement of glycemic control.

4.2.1 Effects of duodenal-jejunal bypass surgery on the function of SGLT1

To investigate whether the activity of SGLT1 is changed after DJB, AMG uptake measurements were performed in different regions of sham and DJB operated rat small intestine of rats with T2LD. I measured phlorizin inhibited uptake of 10 µM AMG in different regions of rats with T2LD three weeks after sham or DJB surgery. The data (Figure 6) indicate that, in the bypassed duodenum and in the jejunum distal to the duodenojejunostomy, phlorizin-inhibited AMG uptake into the enterocytes was significantly reduced by about 50% (P < 0.001 for difference). In ileum, a trend for downregulation of SGLT1 activity was observed, which did not reach significance.

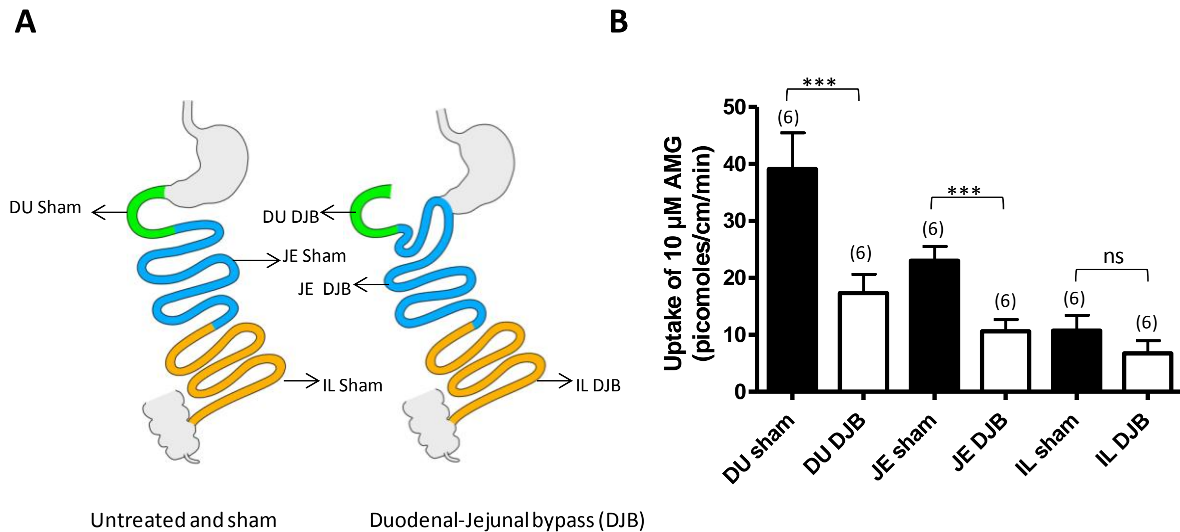

Figure 6: Schematic diagram of the Duodenal-jejunal bypass (DJB) surgery and comparison of SGLT1 activity in small intestinal segments of sham and DJB rats: A) The anatomy after sham and DJB surgery is shown. Duodenum (DU) is indicated in green, jejunum (JE) in blue, and ileum (IL) in yellow. The regions that were analysed for AMG uptake are shown by arrows. B) Small intestinal segments of sham (closed columns) and DJB (open columns) were taken after the animals had been killed in the 3rd week after sham or DJB surgery. Phlorizin inhibitable uptake of 10 µM AMG was measured in different regions of intestine (shown by arrows). Uptake was calculated in terms of picomoles/cm/minute. The numbers of employed animals are given in parenthesis. (***$P < 0.001$ for difference between DU sham and DU DJB, ***$P < 0.001$ for difference between JE sham and JE DJB, ns-not significant, ANOVA with post-hoc Tukey comparison).

4.2.2 Effect of ileal transposition on SGLT1 function and morphological changes of intestine

4.2.2.1 SGLT1 activity in small intestine before and after IT surgery

To investigate whether the transposition of ileal fragment to jejunal region changes the activity of SGLT1, AMG uptake measurements were performed in different regions of small intestine including the jejunal parts close to the transposed ileal segment (See Figure 7A). In sham-operated rats the highest AMG uptake was observed in the duodenum. AMG uptake was similar in the three analyzed jejunal parts of sham operated rats and was lesser than uptake in the duodenum. Ileum showed the least AMG uptake compared to duodenum and jejunum. After IT surgery AMG transport measured in duodenum, in the jejunal part proximal to the transposed ileal segment (JE1 IT in Figure 7A) and in the jejunal part distal to the transposed ileal segment (JE2 IT in Figure 7A) were not changed. AMG uptake in the

ileal part (IL2 IT) was similar to transport measured in the ileal region (IL1 sham) of sham operated rats. Interestingly, AMG uptake of the transposed ileal segment (IL1 IT transposed) was similar to uptake measured in jejunum of sham operated rats. In IL1 IT transposed, AMG uptake per intestinal length was 2.3 fold higher than in IL1 sham (***P < 0.001 for difference). The result suggests that IT surgery does not influence the capacity of small intestine for glucose absorption.

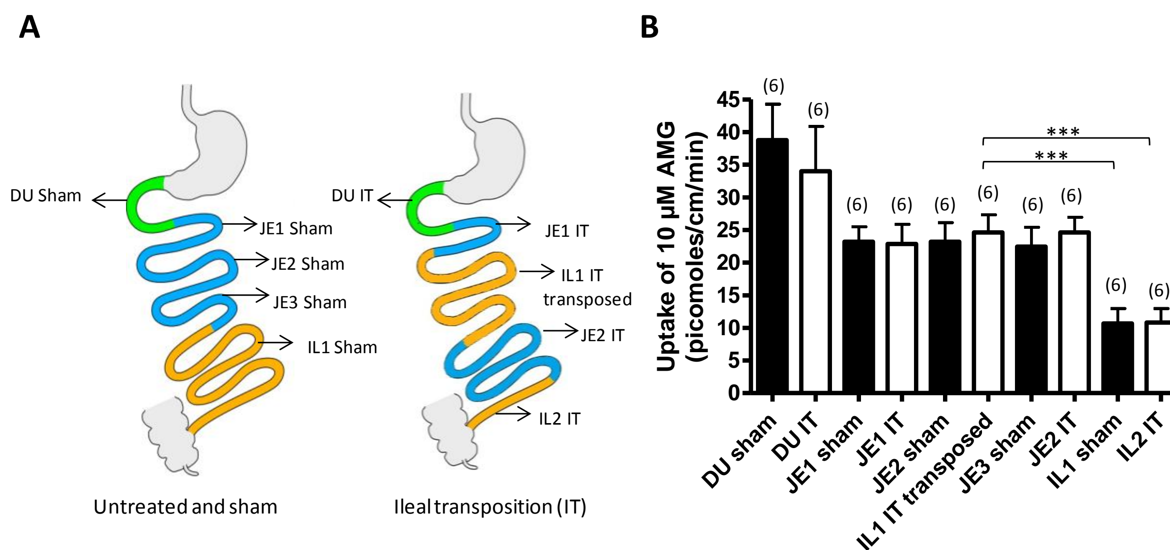

Figure 7: Schematic diagram of the Ileal transposition (IT) surgery and comparison of SGLT1 activity in small intestinal segments of sham and IT rats:: A) The anatomy after sham and IT surgery is shown. Duodenum (DU) is indicated in green, jejunum (JE) in blue, and ileum (IL) in yellow. The regions that were analysed for AMG uptake are shown by arrows. B) Small intestinal segments of sham (closed columns) and IT (open columns) were taken after the animals had been killed in the 5th week after sham or IT surgery. Phlorizin inhibitable uptake of 10 µM AMG was measured in different regions of intestine (shown by arrows). Uptake was calculated in terms of pico moles/cm/minute. The numbers of employed animals are mentioned in parenthesis. (***P < 0.001 difference between IL1 sham and IL1 IT transposed, ***P < 0.001 difference between IL2 IT and IL1 IT transposed, compared by ANOVA with post-hoc Tukey comparison).

4.2.2.2 Morphological and structural changes in the transposed ileum after IT surgery

Morphological changes to the transposed ileal segment after IT surgery were already described earlier (Krawczyk, Wasiutynski et al. 1981, Atkinson, Whipple et al. 1982). To elucidate the morphological and structural changes in IL1 IT transposed, immunohistochemical comparison was performed between IL1 sham and IL1 IT transposed. The data indicate that the diameter of the transposed ileum was increased 1.46 $\pm$ 0.03 times

compared to sham ileum (Figure 8A). In the transposed ileal segment, the length of the villi was increased 1.63 ± 0.02 times (30 determinations, $P < 0.0001$for difference), whereas the width of the villi was not changed (Figure 8B and C).

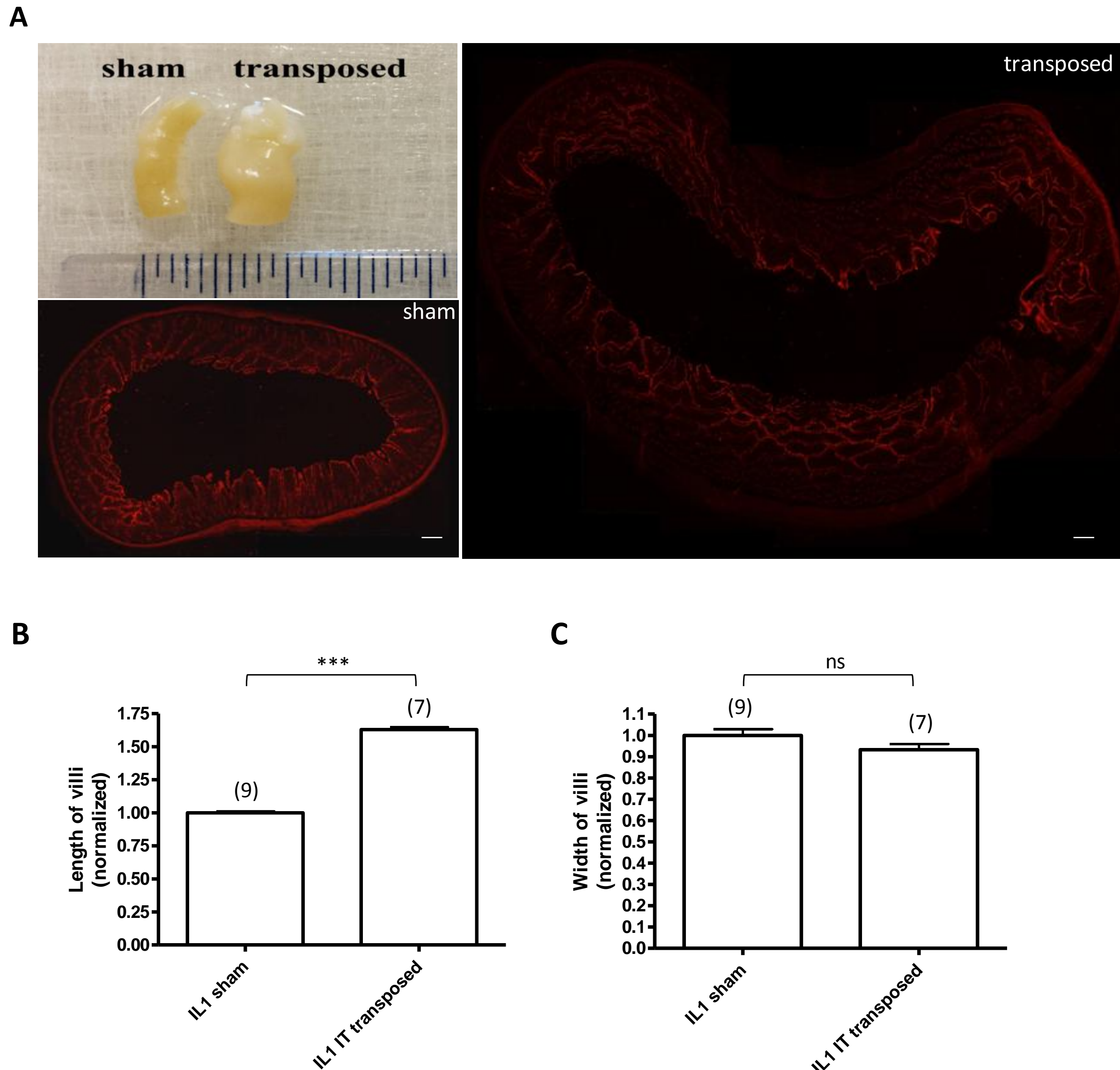

Figure 8: Morphological and structural changes in the transposed ileum: Macroscopic appearance of transposed ileum (A), length and width of the villi in IL1 IT transposed compared to the respective ileal segment IL1 sham (B, C). Samples from IL1 sham and IL1 IT transposed were taken after the animals had been killed in the 5th week after sham or IT surgery. Immunohistochemical analysis was performed using antibody against rat SGLT1. Length and width of the villi were calculated (30 determinations each) from IL1 sham and IL1 IT transposed cross sections. The numbers of the analyzed cross sections are mentioned in parenthesis. In IL1 IT, the length of the villi had increased significantly compared to IL1 sham. (***$P < 0.0001$), whereas the width of the villi did not change ($P = 0.0883$, unpaired t-test). Bar: 50 µm.

4.2.2.3 SGLT1 protein at the luminal membrane of the sham ileum and transposed ileum

Immunohistochemical analysis was performed to check the difference in SGLT1 protein amount at the BBM of the transposed ileum and the respective segment of sham operated rats. Immunostaining of BBM per unit length was measured in these segments. The result shows 2.5 $\pm$ 1.5 fold higher SGLT1 immunostaining intensity in IL1 sham than in IL1 IT transposed (Figure 9A, 80 measurements each). However, because the length of the BBM per cross section was increased about 4.8 fold in IL1 IT transposed compared to IL1 sham (Figure 9B), the amount of SGLT1 protein associated with the BBM showed a 1.9 fold increase in IL1 IT transposed. The similar 2.3 fold increase in AMG uptake (Figure 7B) of IL1 IT transposed suggests that SGLT1 in the BBM of this region is functionally active.

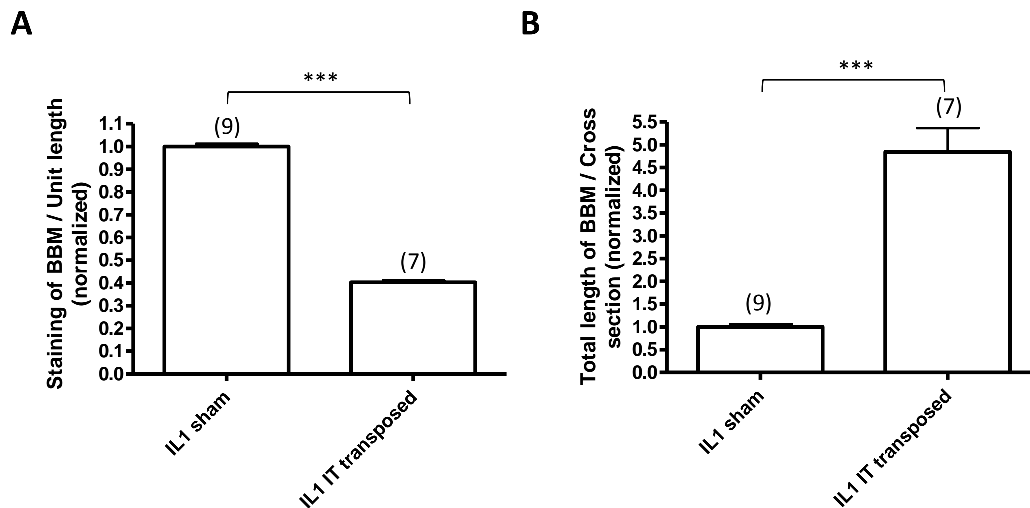

Figure 9: Comparison of SGLT1 protein amount at the BBM of IL1 sham and IL1 IT transposed: Tissue samples from IL1 sham and IL1 IT transposed were taken after the animals had been killed in the 5th week after sham or IT surgery. Immunohistochemical analysis was performed using antibody against rat SGLT1. Staining of the BBM per unit length (A) and the total length of the BBM per cross section (B) were calculated. The numbers of the analyzed cross sections are given in parenthesis. Significant increase in BBM staining per unit length (***P < 0.0001, 80 measurements each) was observed in IL1 sham than in IL1 IT transposed. The length of the BBM per cross-section in IL1 IT transposed was increased significantly (***P < 0.0001 for difference between IL1 sham and IL1 IT transposed, unpaired t-test).

4.2.2.4 GLP-1 secreting L-cells in the mucosa of sham and transposed ileum

L-cells secrete enteroharmone GLP-1 that influence glycemic control. In rats after IT surgery, increased GLP-1 levels were observed after gavage with glucose (Jurowich et al., unpublished data). To determine whether the number of L-cells in the mucosa of transposed ileum was changed, we performed immunohistochemical analysis using antibody against GLP-1. GLP-1 positive L-cell number was counted per cross section and compared between IL1 IT transposed and IL1 sham. In IL1 sham, 9.2 $\pm$ 2.5 L-cells per cross section and in the IL1 IT, 17.5 $\pm$ 1.2 L-cells per cross section were observed (15 determinations each). The data indicate that there is a significant increase in L-cell number in IL1 IT (**P = 0.0057 for difference) that contributes to the higher GLP-1 secretion.

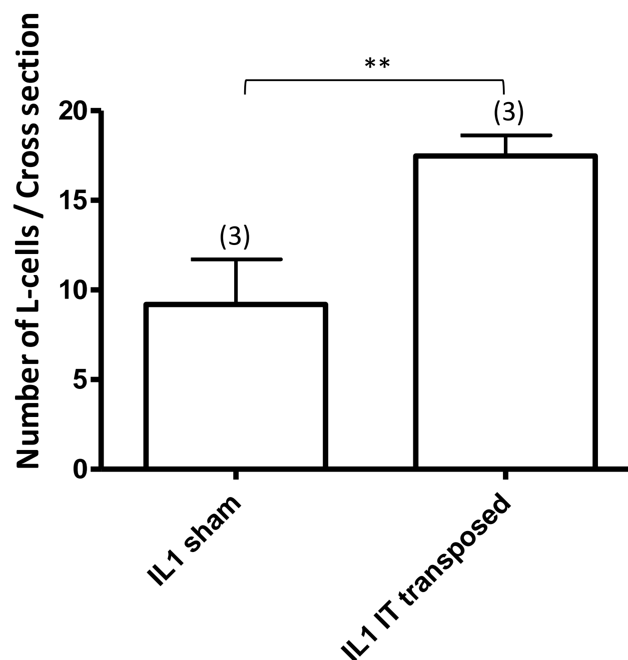

Figure 10: Comparison of L-cell number in the mucosa of IL1 sham and IL1 IT transposed: Transposed ileum (IL1 IT) and the corresponding ileal fragment of sham-operated animals (IL1 sham) were taken after the animals had been killed in the 5th week after sham or IT surgery. Immunohistochemical analysis was performed using antibody against GLP-1. Immunoreactive L-cell numbers was counted per cross section and compared between IL1 sham and IL1 IT transposed (15 determinations each). The numbers of employed animals are shown in parenthesis. Significant increase in GLP-1 positive cells was seen in transposed ileum compared to IL1 sham (**P = 0.0057, unpaired t-test).

4.3 Regulation of SGLT1 activity in human and mice small intestine by tripeptides derived from the regulatory protein RS1

4.3.1 Regulation of human intestinal SGLT1 activity by RS1 derived peptides

Characterization studies of RS1 revealed that tripeptides derived from RS1 down-regulate SGLT1 mediated AMG uptake in *Xenopus laevis* oocytes (Vernaleken, Veyhl et al. 2007). Two tripeptides Gln-Cys-Pro (QCP) and Gln-Ser-Pro (QSP) were identified to induce post transcriptional down-regulation of the exocytotic pathway of hSGLT1 at the TGN (Veyhl et al. 2006, Kroiss et al. 2006). To investigate the regulation of SGLT1 by RS1 in small intestine, I investigated the effect of these tripeptides in jejunal segments of human and mice (*ex vivo*). Human jejunal tissues were obtained from the patients who underwent bariatric operations. AMG uptake measurements were performed after pre-incubating the tissue for 30 minutes in DMEM medium in presence of 5 mM glucose with or without 5 mM of QSP, thiophosphorylated QSP (QSpP), QEP (glutamate mimicks phosphorylation of serine) and several other tripeptides where serine in QSP was replaced by other amino acids. The results are shown in Figure 11. They show that QEP and QSpP down-regulated phorizin inhibited AMG uptake mediated by SGLT1 in human jejunum (***$P < 0.001$ and *$P < 0.05$ respectively). Downregulation of SGLT1 mediated AMG uptake by QEP was more pronounced than downregulation by QSpP.

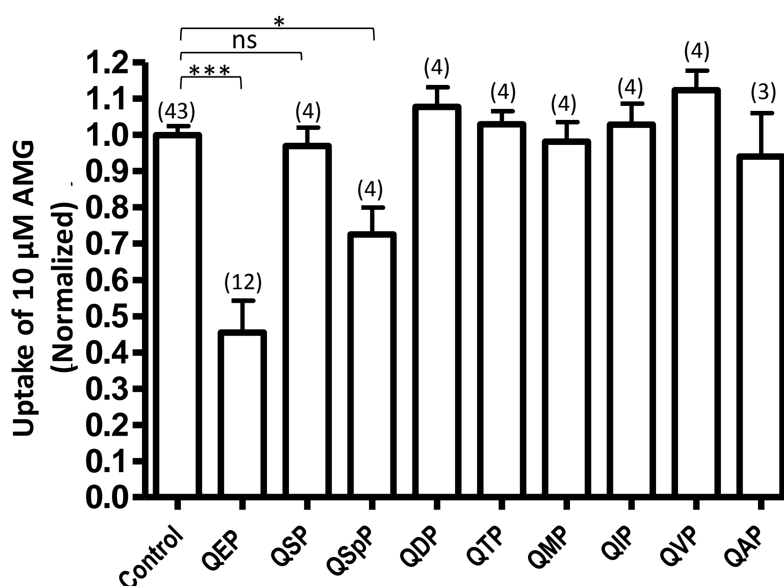

Figure 11: Effect of ex vivo Incubation of human jejunal mucosa with tripeptides derived from RS1: Mucosa layers were isolated from segments of human jejunum obtained during bariatric operations. Circular areas of identical size were taken and were incubated for 30 min without (control)

and with 5 mM RS1 derived tripeptides in presence of 5 mM glucose. After incubation extracellular glucose and tripeptides were removed by washing and phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. The numbers of employed human jejunal tissue samples are mentioned in parenthesis. Among the tested peptides, QEP and thiophosphorylated QSP significantly down-regulated AMG uptake (***P < 0.001 difference between control and QEP treatment, *P < 0.05 difference between control and QSpP treatment, ANOVA with post-hoc Tukey comparison).

4.3.2 Dose dependent effect of QEP, QSpP and QSP on SGLT1 activity in human jejunum

To obtain more information about the affinities of QEP, QSpP and QSP, I focused on these peptides and investigated the concentration dependent regulation. The phlorizin inhibitable 10 µM AMG uptake measurements were performed with and without different concentrations of QEP, QSpP and QSP. The results show that QEP inhibited SGLT1 activity significantly even at low (1 mM) concentration (Figure 12 A, ***P < 0.01 for difference). But higher concentration of QSpP (5mM) and QSP (15mM) was needed for the downregulation (Figure 12 B and C). Figure 12 A indicates that QEP showed dose dependent downregulation of SGLT1 activity. At 15 mM concentration QEP and QSpP inhibited SGLT1 activity by 73% and 42% respectively (***P < 0.01 for difference). Since QEP showed the highest affinity among the tested tripeptides, it was confirmed as the effective peptide.

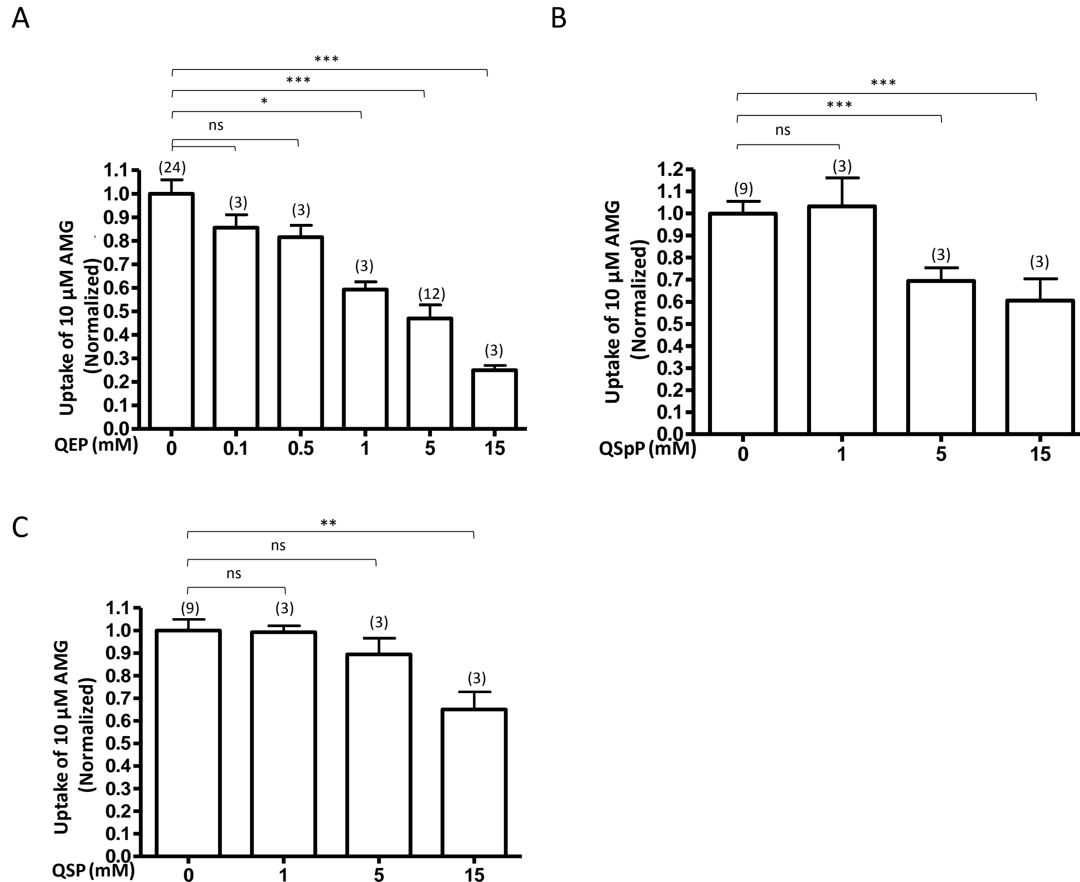

Figure 12: Dose dependent effect of tripeptides QEP, QSpP and QSP on transport activity of SGLT1 in human jejunum: Mucosa layers were isolated from segments of human jejunum obtained during bariatric operations. Circular areas of identical size were taken and were incubated for 30 min without and with different concentrations of QEP (A), QSpP (B) or QSP (C) in presence of 5 mM glucose. After washing phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. The numbers of employed human jejunal tissue samples are indicated in parenthesis. Among the tested tripeptides QEP showed dose dependent inhibition of SGLT1 activity (*P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with post-hoc Tukey comparison).

4.3.3 Regulation of SGLT1 activity in wild type and RS1 KO mice by RS1 derived tripeptides

The luminal glucose concentration plays key role in the regulation of SGLT1. The data obtained from mice (Alexandra Friedrich, unpublished data) suggest that QSP and QEP do not down-regulate SGLT1 activity in wild type mice when the luminal glucose concentration is low, as the endogenous RS1 is active under this condition. It is expected that at high glucose concentration, SGLT1 activity increases as endogenous RS1 is inactive. To

understand the functioning of tripeptides at high luminal glucose concentration where the endogenous RS1 is inactive, I investigated the regulation of SGLT1 activity by our favourite peptides (QEP, QSP and QSpP) at high glucose concentration.

Phlorizin inhibited uptake of 10 µM AMG was measured in the jejunal segments of wild type and RS1 KO mice after pre incubation for 30 minutes with and without 5 mM QEP, QSP and QSpP in presence of high glucose concentration. The data (Figure 13) indicate that at high glucose concentration, QEP and QSpP down-regulated SGLT1 activity in both wild type and RS1 KO mice equally and the removal of RS1 (RS1 KO) did not impact the regulation by external RS1 tripeptides.

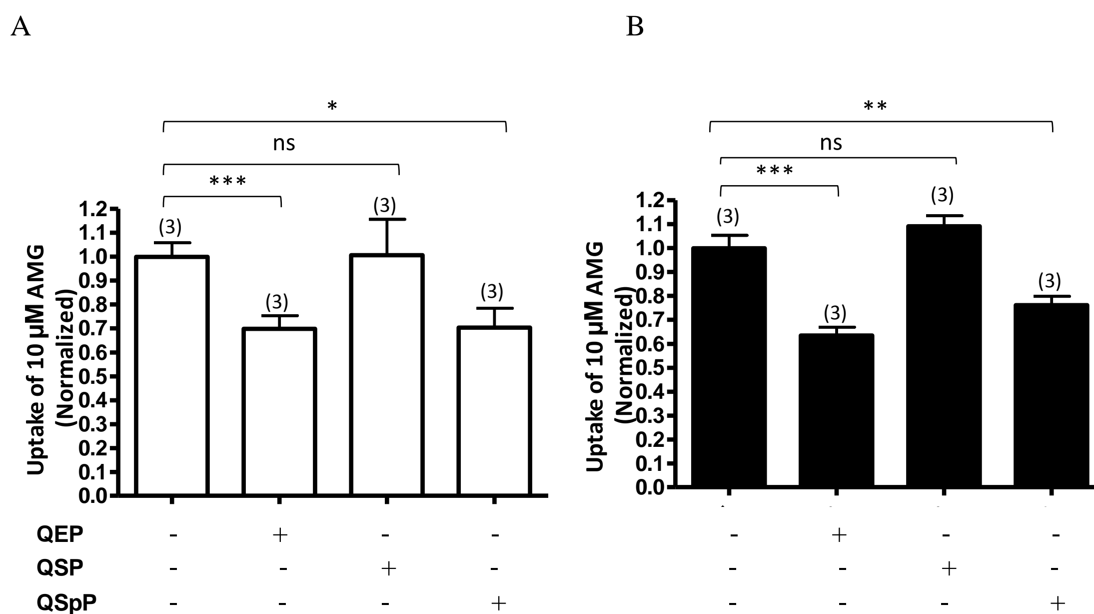

Figure 13: Effect of tripeptides QEP, QSP and QSpP on transport activity of SGLT1 in wild type and RS1 KO mice: Wild type mice (A) and RS1 KO mice (B) mice were starved for 18 hours prior to measuring SGLT1 mediated AMG uptake. Everted Jejunal segments were taken and incubated for 30 min without and with 5 mM tripeptides QEP, QSP and thiophosphorylated QSP in presence of 5 mM glucose. After washing phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. The numbers of employed animals are mentioned in the parenthesis. 5 mM concentration of QEP and thiophosphorylated QSP significantly down-regulated SGLT1 activity but QSP did not show any downregulation. Absence of endogenous RS1 did not impact the regulation. (ns-not significant, ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, calculated by ANOVA with post-hoc Tukey comparison).

4.4 Identification of SGLT1 activity in human colon and its regulation by tripeptide QEP

Previous studies in mice revealed the existence of glucose transporters in the large intestine. Immunohistochemical analysis using the anti-SGLT1 antibody confirmed the expression of SGLT1 protein in colon (Yoshikawa, Inoue et al. 2011). Figure 2 also confirms the presence of SGLT1 dependent AMG uptake in mouse colon. Here, I investigated the SGLT1 transport activity in human colon tissues that were taken from patients during colitis surgery. AMG uptake measurements were performed using identical areas of the dissected mucosa. The result confirmed the existence of SGLT1 activity on the luminal surface of enterocytes in colon.

To further understand the regulation of QEP on SGLT1 activity in human colon, uptake measurements were performed without and with QEP. A significant downregulation of SGLT1 mediated uptake in human colon (Figure 14) was obtained after 30 minutes incubation with 15 mM QEP (**P = 0.0043). The result suggests that QEP is capable to downregulate SGLT1 activity in human colon as well.

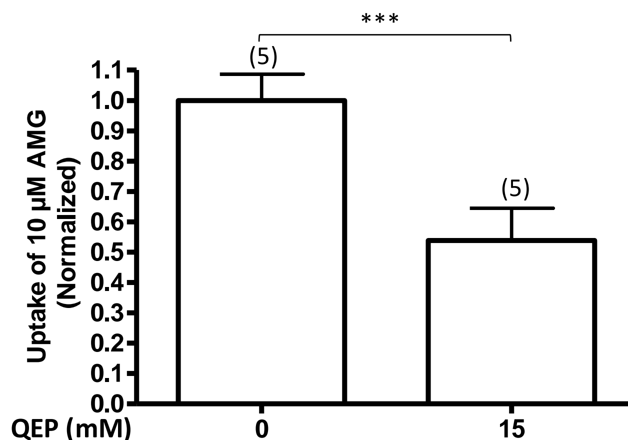

Figure 14: Downregulation of SGLT1 activity in human colon in presence of 15 mM QEP: Mucosa layers were isolated from segments of human colon obtained during colitis operations. Circular areas of identical size were taken and were incubated for 30 min without and with 15 mM QEP in presence of 5 mM glucose. After washing phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. The numbers of employed human colon tissue samples are shown in parenthesis. Human colon showed SGLT1 mediated AMG uptake and was significantly inhibited by 15 mM QEP (**P = 0.0043 by unpaired t-test).

4.5 Effect of tripeptide QEP on SGLT1 activity in different animal models

RS1, a regulatory protein is present in different mammals (Veyhl, Spangenberg et al. 1993, Lambotte, Veyhl et al. 1996, Reinhardt, Veyhl et al. 1999, Osswald, Baumgarten et al. 2005). To establish the biomedical importance of QEP as an essential SGLT1 regulator, it is of further interest to understand the effect of this tripeptide for SGLT1 regulation in different species. To have maximum regulation by QEP, higher concentration i.e. 15 mM of the tripeptide was used. Intestinal tissues were collected from different animal models like wild type mice, RS1 KO mice, Leptin KO mice (ob/ob mice), Rabbit, Pig and Human. AMG uptake measurements were performed with and without 15 mM QEP. The data indicate that 15 mM QEP effectively down-regulated SGLT1 activity in all animal models (***$P < 0.001$) and QEP is the main peptide to be focused on.

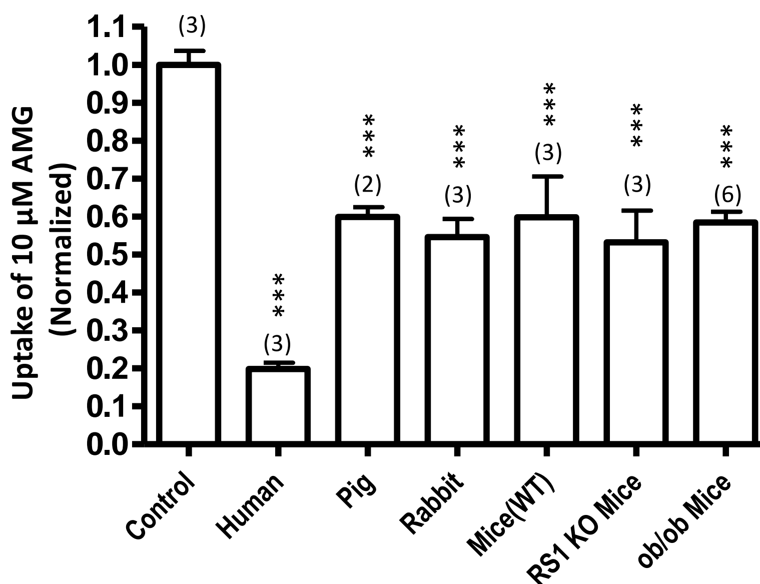

Figure 15: Effect of tripeptide QEP (15 mM) on transport activity of SGLT1 in different animal models: Everted jejunal segments in mice and rabbit, identical areas of mucosa in pig and human jejunum were taken and incubated for 30 minutes without (control) and with 15 mM QEP in presence of 5 mM glucose. After washing phlorizin inhibited uptake of 10 µM AMG was measured. Uptake values are normalized to 1. The numbers of employed animals or human jejunal tissue samples are mentioned in the parenthesis. Significances of differences were calculated by ANOVA with post-hoc Tukey comparison. QEP significantly down-regulated SGLT1 activity in the small intestine of wild type mice, RS1 KO mice, ob/ob mice, rabbit, pig and human (***$P < 0.001$).

5. Discussion

In recent years, novel approaches for the treatment of type 2 diabetes suggest that altering glucose absorption in the small intestine might be a possible strategy to control blood glucose levels. Bariatric surgery has shown promising results in controlling diabetes (Brolin 2002, Christou, Sampalis et al. 2004, Steinbrook 2004). In this study an attempt was made to understand the impact of two types of bariatric procedures i.e. DJB and IT on rat intestinal glucose transport mediated by SGLT1. We first demonstrated that DJB and IT improved diabetes in a non obese type 2 diabetes animal model independent of weight loss. We then investigated the involvement of SGLT1 regulation which is crucial for small intestinal glucose absorption in controlling diabetes by DJB and IT.

The present modified DJB procedure (without Roux-Y reconstruction) was performed to exclude undesired effects of the Roux-Y limb and to minimize surgical stress and trauma. The observed positive effects of DJB confirms the existing data of several groups showing improved glycemic control in genetically diabetic Goto-Kakizaki rats and non-obese rats with STZ-induced diabetes (Pacheco, de Luis et al. 2007, Kindel, Yoder et al. 2009, Breen, Rasmussen et al. 2012). Although some previous studies in rats with diet induced obesity duodenal bypass did not show improvement in insulin resistance (Kindel, Martins et al. 2011), in our study, improved glycemia has been demonstrated in experimental animal models with reduced insulin sensitivity which mimic the clinical feature of human type 2 diabetes mellitus. In this animal model combination of HFD and low dose of streptozotocin was used to induce type 2 diabetes (Srinivasan, Viswanad et al. 2005, Zhang, Lv et al. 2008).

To determine whether SGLT1-mediated glucose absorption in the small intestine is changed after bariatric surgery, the complex regulation of SGLT1 expression in the small intestine must be taken into account. These include modification in expression of SGLT1 within different intestinal regions, sex dependent regulation, up-regulation after RS1 removal and upon diabetic disease state. Considering this complex situation, we determined the SGLT1 activity in different parts of the intestine, in male and female, in wild type and RS1 KO mice and also compared its activity in non-diabetic and type 2 diabetic individuals. Previous studies in mice revealed the mRNA expression pattern of SGLT1 throughout the gastro intestinal tract (Yoshikawa, Inoue et al. 2011). Our study provided more specific information about the SGLT1 activity pattern in different regions of intestine (Figure 2). Duodenum showed the higher SGLT1 activity followed by jejunum and ileum. Along with understanding

the SGLT1 activity, this study also indirectly provided more specific information about the localization of SGLT1 in different regions of small intestine, which regulates glucose absorption. Interestingly some amount of SGLT1 activity was seen in the large intestine, suggesting the existence of the physiological uptake of glucose in the large intestine that may help in nourishment for enterocytes. In our study, we also compared the SGLT1 activity between male and female and the study revealed that SGLT1 activity in the small intestine is not sex dependent. The same observation was recently reported by our co-workers in the human kidney (Vrhovac, Balen Eror et al. 2014). Studies in rats and humans showed that diabetic condition elevated SGLT1 protein expression in the small intestine (Debnam, Karasov et al. 1988, Burant, Flink et al. 1994, Dyer, Wood et al. 2002). Our results showing the elevated SGLT1 activity upon type 2 diabetic condition support these findings (Figure 5). Up-regulation of SGLT1 activity during diabetes may be explained by an increased D-glucose concentration in the enterocytes due to increased blood glucose and basolateral uptake via GLUT2.

In an attempt to identify the region of intestine involved in glycemic control after DJB, we investigated SGLT1 transport activity in different regions of the small intestine before and after surgery. After DJB surgery, the SGLT1 mediated transport in duodenum, jejunum distal to the duodeno-jejunostomy and ileum were decreased (Figure 6). The SGLT1 mediated glucose uptake in jejunum, which was doubled in HFD STZ (diabetic) rats, was normalized to the level of healthy animals. The mechanism of this downregulation is unclear. It may be due to less hydrolysis of polysaccharides in the small intestine. This could also be due to impaired parasympathetic innervation. Studies reported that insulin in the portal vein stimulated SGLT1 mediated small intestinal glucose absorption via parasympathetic nerves (Stumpel, Kucera et al. 1996). Reduction of glucose absorption after DJB due to removal of the duodenum and proximal jejunum may lead to reduced serum glucose levels and thereby improve diabetic control. The changes in SGLT1 activity could contribute to positive effects on diabetes along with the release of gut hormones that regulate insulin secretion (Lovshin and Drucker 2000, Thorens 2003). It states that the activity of SGLT1 in the BBM of enterocytes determines the velocity of small intestinal glucose absorption (Gorboulev, Schurmann et al. 2012). The absorbed glucose in the blood stimulates pancreatic insulin secretion in a combined action with insulinotropic enterohormones (Preitner, Ibberson et al. 2004). It is possible to hypothesize that the impaired glucose absorption in duodenum and jejunum after DJB leads to increased glucose levels in the ileum. This might in turn increase

the glucose-dependent stimulation of enteroharmone GLP-1 secreted by L-cells that are mainly located in the ileum (Lovshin and Drucker 2000).

I also investigated the effects of ileal transposition (IT) surgery on small intestinal SGLT1 mediated glucose transport. IT does not change the regulatory mechanisms governed by stomach as it does not include restriction of stomach size and/or alimentary path. In type 2 diabetes induced rats, IT surgery animals showed improved glycemia independent of weight loss (Jurowich et al., unpublished data). We studied whether the SGLT1 mediated small intestinal glucose absorption was changed after IT because altered glucose absorption may influence the serum glucose and may alter glucose dependent insulin secretion. The result (Figure 7) suggests that the activity of SGLT1 is not changed after IT surgery. However, the SGLT1 uptake in the transposed segment per unit length was increased to a value similar to the uptake value in the jejunum of sham rats. But the rate of SGLT1 uptake remained largely unchanged in transposed ileal segment. The unchanged SGLT1 activity after IT surgery indicate that changes in SGLT1 mediated glucose absorption do not participate in the improved weight independent glycemic control after IT surgery.

Several studies have reported on increased plasma GLP-1 levels in IT operated animals. GLP-1 is a potent insulinotropic hormone that improves glucose tolerance (Patriti, Facchiano et al. 2005, Strader, Vahl et al. 2005). It has also been reported that IT in non obese type 2 diabetic rats controlled diabetes by increasing GLP-1 (Wang, Hu et al. 2008). Gaitonde et al reported that the improvement in OGTT after IT was abolished in presence of GLP-1 receptor antagonist (Gaitonde, Kohli et al. 2012). The obtained results are in accordance with the previous findings. We also observed an increase in the secretion of GLP-1 in response to glucose gavage after IT surgery (Jurowich et al., unpublished data). Considering these reports it can be stated that elevated secretion of GLP-1 is probably involved in the body weight-independent therapeutic effect of IT surgery on diabetes. After IT surgery, the terminal ileum (ileal segment) containing most of the L-cells is situated within the proximal jejunum where it is exposed to high glucose concentration. This situation could stimulate the secretion of GLP-1 which enhances insulin secretion.

Bariatric surgery can change intestinal morphological structure, may be due to surgery induced impairment of innervation and altered nutrient signals that impact the expression of transporters. Morphological assessment after RYGB and ileo-jejunal transposition revealed the changes in villus height and crypt depth (Robinson JW et al.1978, Stearns, Balakrishnan

et al. 2009). Previous studies have reported on increased weight, diameter, protein content and DNA content of mucosa in the transposed ileal segment (Ulshen and Herbst 1985). In our study transposed ileum segment has been observed with increased diameter. We also observed the increase in thickness of the bowel wall (Figure 8). Immunostaining with SGLT1-Ab revealed that the length of the BBM per cross section had increased and so has the length of villi, but the width of the villi did not change significantly (Figure 8). However, these changes in villi length and diameter cannot clearly explain the positive effect of IT. Enterohormone GLP-1 that regulates insulin is secreted by L-cells (Lovshin and Drucker 2000). Our immunohistochemistry data showed 1.9 fold increase in the number of GLP-1 secreting L-cells in the transposed ileum compared to sham ileum (Figure 10). This increase in L-cell number may contribute to the increased GLP-1 secretion.

SGLT1 regulation has been shown to play a major role in the glycemic control of diabetes. Our study also reveals that improvement of glycemic control by bariatric surgery involves SGLT1. To further draw light on SGLT1 regulation, we also focused on RS1 derived tripeptides that involve in the downregulation of SGLT1. *In vitro* studies in *Xenopus laevis* oocytes showed that tripeptides derived from the protein RS1 mediate short term down regulation of SGLT1 activity (Vernaleken, Veyhl et al. 2007). To determine whether oral application of these peptides can be used for downregulation of SGLT1 mediated glucose absorption after glucose rich meals, we investigated the regulation of SGLT1 activity by different RS1 derived tripeptides in mouse and human jejunal tissues *ex vivo* at high glucose concentrations.

We showed that tripeptides QEP and QSpP are capable of down-regulating SGLT1 activity in mice and human jejunum (Figure 12 and 13). The study also revealed that the affinity of QEP to down-regulate SGLT1 mediated glucose absorption is higher than other tripeptides. The biomedical importance of QEP was proved by its concentration dependent inhibition of SGLT1 activity. It was of further interest to understand the role of QEP for SGLT1 regulation in different species. The result (Figure 15) suggests that QEP is able to down-regulate SGLT1 activity in human, pig, rabbit and mouse which proved the universal role of QEP in SGLT1 regulation.

RS1 is critically involved in the glucose dependent short term up-regulation of SGLT1 (Maike Veyhl-Wichmann and Helmut Kipp, unpublished data). When the glucose concentration in small intestine is low, RS1 down-regulates the delivery of SGLT1 from the TG to

the plasma membrane. After meals, glucose levels are increased in the small intestine and RS1-mediated down-regulation of SGLT1 delivery to the BBM is weakened. Under this condition glucose binds to the RS1-Reg receptor and induces dissociation of RS1-Reg from its receptor. This leads to clearance of receptor mediated blockage of vesicle release from the TG, and results in up-regulation of SGLT1 in the BBM. The data of Alexandra Friedrich suggest that QEP and QSP do not downregulate SGLT1 activity in wild type mice when the luminal glucose concentration is low. Based on the results obtained by our colleagues, we hypothesize that when the glucose concentration in small intestine is low, the endogenous RS1 is active, down-regulates SGLT1 expression and further inhibition by the RS1 derived peptides is not possible. At higher glucose concentration, the endogenous RS1 is inactive and the peptides are supposed to bind to RS1-Reg receptor and block the release of SGLT1 containing vesicles at TG. This hypothesis is supported by the result (Figure 13) showing that QEP and QSpP down-regulated SGLT1 activity equally in wild type and RS1 KO mice at high luminal glucose concentration.

Tripeptides QEP and QSpP that down-regulate the SGLT1 transport activity are substrates of the peptide transporter PepT1 which help them to pass from intestinal lumen to enterocytes. Thereby we can state that identification of tripeptides that control the glucose dependent up-regulation of glucose absorption provides a new target for treatment of type 2 diabetes.

All these investigations concerning DJB and RS1 derived tripeptides suggest that if SGLT1 transport activity is inhibited in the small intestine, secretion of antidiabetic enterohormone GLP-1 should be increased. After DJB surgery or oral application of tripeptides SGLT1 transport activity is inhibited in the proximal intestine, decreased glucose absorption in the proximal part leads to increased GLP-1 secretion because the glucose concentration in the ileum is increased. L-cells which are mainly located in the ileum secrete GLP-1 in response to glucose and short chain fatty acids (Psichas, Sleeth et al. 2014, Kuhre, Frost et al. 2015). In case of IT surgery the transposed ileal segment containing increased number of L-cells is exposed to more glucose, this could help in the increased secretion of GLP-1.

In the present study, we showed that DJB surgery decreases glucose absorption in small intestine by down-regulation of SGLT1 transport activity. However, IT shows no significant effect on SGLT1 transport activity. The data suggest that increased GLP-1 secretion after DJB and IT is key for the positive effect on diabetes. The modified model of DJB without reconstruction by Roux-Y and IT are feasible and appear to be promising models that can be

applied to treat type 2 diabetes in non obese or slightly overweight patients. We also showed that tripeptides QEP and QSpP down-regulate SGLT1 activity in small intestine of mice and humans in presence of high glucose. Therefore, oral application of RS1 derived tripeptides QEP and QSpP can be used for treatment of type 2 diabetes. The present study offers a new aspect of SGLT1 regulation by different bariatric procedures and RS1 derived tripeptides.

6. Summary

Bariatric surgery represents the first-line treatment for morbid obesity, resulting in weight loss and improved diabetes control. The positive effect of bariatric surgery on type-2 diabetes is unclear. Increased secretion of insulin regulating enterohormone glucagon-like-peptide 1 (GLP-1) has been observed in rats with experimental type 2-like diabetes following duodenal-jejunal bypass (DJB) and ileal transposition (IT). Sodium dependent glucose co-transporter (SGLT1) is involved in the secretion of GLP-1 that in turn regulates insulin secretion. In the present study, an attempt was made to elucidate the impact of DJB and IT on SGLT1 mediated glucose transport. Transport measurements using phlorizin inhibited uptake of SGLT1-specific glucose analogue [^{14}C]α-Methyl-D-glucopyranoside (AMG) were performed to determine the changes in SGLT1 transport upon these surgical procedures. DJB surgery down-regulated SGLT1-mediated glucose absorption in small intestine which contributes to the body-weight independent improvement of type 2 diabetes. However, IT surgery did not change the SGLT1-mediated glucose transport. Immunohistochemical analysis revealed that in IT, the transposed ileum showed increased diameter, increased villi length and increased number of GLP-1 secreting L-cells. The weight-independent improvement in glycemic control after IT is not related to SGLT1-mediated glucose absorption but may be linked to increased GLP-1 secretion.

Additionally, the study also focused on the regulation of SGLT1 by several RS1 derived tripeptides in mouse and human intestinal tissues (*ex vivo*). Phlorizin inhibited uptake of AMG was measured without and with tripeptides. QEP and thiophosphorylated QSP down-regulated SGLT1 activity in small intestine in a concentration-dependent manner. Among the tested tripeptides, QEP showed higher activity and further analysis in various species demonstrated its universal role in SGLT1 regulation. The data thus indicate that RS1 derived tripeptides QEP and thiophosphorylated QSP may be employed for the treatment of type 2 diabetes.

7. Zusammenfassung

Bariatrische Operationen repräsentieren die Behandlung erster Wahl bei krankhafter Fettleibigkeit, resultierend in Gewichtsverlust und verbesserter Diabetes-Kontrolle. Der positive Effekt bariatrischer Operationen auf den Typ-2 Diabetes ist unklar. Erhöhte Sekretion von Insulin, welches das Enterohormon Glucagon-like-peptide 1 (GLP-1) reguliert, wurde beobachtet bei Ratten mit experimentellem Typ 2-ähnlichem Diabetes nach duodenalem-jejunalem Bypass (DJB) und ilealer Transposition (IT). Der Natrium-abhängige Glucose Cotransporter (SGLT1) ist beteiligt an der Sekretion von GLP-1, das wiederum die Insulin-Sekretion reguliert. In der vorliegenden Studie wurde der Versuch unternommen, die Bedeutung von DJB und IT für den durch SGLT1 vermittelten Glucose-Transport aufzuklären. Transportmessungen der durch Phlorizin hemmbaren Aufnahme des SGLT1-spezifischen Glucose-Analogs [^{14}C]α-Methyl-D-glucopyranosid (AMG) wurden durchgeführt, um die durch diese chirurgischen Eingriffe bedingten Änderungen des Transports durch SGLT1 zu bestimmen. Die Daten deuten darauf hin, dass DJB die SGLT1-vermittelte Glucose Absorption im Dünndarm verringert, was zu einer körpergewichts-unabhängigen Verbesserung des Diabetes Typ 2 beiträgt. Aber IT veränderte den SGLT1-vermittelten Glucose-Transport nicht. Immunhistochemische Analysen zeigten, dass bei IT das transponierte Ileum einen vergrößerten Durchmesser, eine erhöhte Länge der Villi und eine erhöhte Anzahl der GLP-1 sekretierenden L-Zellen aufwies. Die gewichtsunabhängige Verbesserung der glykämischen Kontrolle nach IT steht nicht im Zusammenhang mit der durch SGLT1-vermittelten Glucose-Absorption, sondern könnte verbunden sein mit einer erhöhten GLP-1 Sekretion.

Damit einhergehend fokussiert sich die Studie auch auf die Regulation des SGLT1 durch verschiedene, von RS1 abgeleitete Tripeptide in Darm-Gewebe von Maus und Mensch (*ex vivo*). Die phlorizin-hemmbare Aufnahme von AMG wurde gemessen mit und ohne Tripeptide. QEP und thiophosphoryliertes QSP regulierten die SGLT1-Aktivität herunter im Dünndarm auf eine konzentrationsabhängige Weise. Unter den getesteten Tripeptiden zeigte QEP eine höhere Aktivität und weitere Analysen in verschiedenen Spezies zeigten seine universelle Rolle in der SGLT1-Regulation. Die Daten zeigen daher, dass die von RS1 abgeleiteten Tripeptide QEP und thiophosporyliertes QSP eingesetzt werden könnten zur Behandlung von Typ2 Diabetes.

8. Abbreviations

AMG	Alpha Methyl D-Glucopyranoside
BBM	Brush border membrane
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CNT	Concentrative nucleoside transporter
DJB	Duodenal- Jejunal Bypass
DMEM	Dulbecco`s modified Eagle`s medium
DU	Duodenum
GIP	Glucose-dependent insulinotropic peptide
GK	Goto-Kakizaki
GLP-1	Glucagon like peptide-1
GLUT	Facilitated glucose transporters
HFD	High fat diet
IL	Ileum
IT	Ileal Transposition
JE	Jejunum
NIH	National Institutes of Health
OCT	Organic cation transporter
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PKC	Protein Kinase C
PME	Plasma membrane enriched
QAP	Gln-Ala-Pro
QDP	Gln-Asp-Pro
QEP	Gln-Glu-Pro
QIP	Gln-Ile-Pro
QMP	Gln-Met-Pro
QSP	Gln-Ser-Pro
QTP	Gln-Thr-Pro
QVP	Gln-Val-Pro
RS1 KO	RS1 knockout
RT	Room temperature

RYGB	Roux-en-Y gastric bypass
SEM	Standard Error of Mean
SGLT	Na^+-D-glucose co-transporter
SLC5A	Solute carrier family
STD	Standard diet
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
TGN	Trans Golgi Network

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Affidavit

I hereby declare that my thesis entitled "Regulation of the Na^+-D-glucose cotransporter SGLT1 in small intestine in response to bariatric surgery and peptides derived from protein RS1 (*RSC1A1*)" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Hyderabad, 30.05.2015 Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation Regulation des Na^+-D-Glucose Kotranporters SGLT1 im Dünndarm nach bariatrischen Operation und durch von Protein RS1 (RSC1A1) abgeleitete Peptide eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Hyderabad, 30.05.2015 Unterschrift